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Renaturation of Intramolecularly Cross-linked Bovine RNase A

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Renaturation experiments of intramolecularly cross-linked reduced RNase A (Lys^{7}-Lys^{37} or Lys^{31}-Lys^{37}), in which a part of the polypeptide conformation of the enzymatically active form should be maintained by the presence of a cross-linkage, showed that there was no enhancement of renaturation rate in comparison with the non-modified protein. Recovery of an enzyme activity was only 75% after 24 hrs oxidation. Oxygen accelerated the renaturation reactions in the same amount for both of the proteins, but the effect of preincubation of the reduced proteins in the renaturation condition except the absence of oxygen was not observed.

INTRODUCTION

During the last decade the idea of a protein folding was fairly well established, though still limited in phenomenological aspects. Almost all proteins, with or without subunits, have been shown to be able to renature from artificially denatured states in which the original conformations were completely lost. Time courses of renaturation reactions have been also reported in several cases: most cases agreed in results that the processes contained rapid spectral (UV, CD, and ORD) changes followed by a slow enzyme activity expression. Especially RNase A, a ribonuclease in a bovine pancreas, has attracted attentions of many workers and actually many interesting fundamental results on protein renaturation have been piled up with this enzyme, including the effects of side chain modifications, kinetics, renaturation activating enzyme, and others (see ref. 1 as a review on these topics). No experiments, however, have been carried out on the effects of the original protein conformation partially hold in a starting denatured state. Our interests on the fundamental nature of protein renaturation are asking: will the presence of a part of the original conformation of a native protein polypeptide chain favor the renaturation process? Freezing a part of conformational freedoms will be achieved by an introduction of an intramolecular cross-linkage linking fairly close regions (say, within 10 residues) of a polypeptide chain. Cross-linked RNase A (Lys^{7}-Lys^{37} or Lys^{31}-Lys^{37})[2] would serve as an adequate model for this purpose. This paper deals with a renaturation experiment of this modified protein.

EXPERIMENTAL

The sample of intramolecularly cross-linked RNase A used in this experiment was the same as described in the preceding paper.[3] Pure guanidinium chloride was prepared from guanidinium carbonate. The method for preparation of pure guanidinium chloride have

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been described by several authors,[4] but slight modifications were introduced in the present study. Commercial guanidinium carbonate was recrystallized from water, converted into the chloride with conc. hydrochloric acid. The solution was treated with Norit A at pH 9, carefully filtrated, and concentrated. Absorbance of the solution of the guanidinium chloride (2 g in 2 ml water) prepared in this method was 0.06 at 230 nm. DTNB (dithiobis-2-nitrobenzoic acid) was supplied from Aldrich Chem. Co. All other reagents were of reagent grade, and used without further purification.

Reduction of disulfide bonds were achieved by 2-thioethanol in the presence of urea.[5] Sulfhydryl groups of the protein were titrated spectrophotometrically by the use of DTNB in the following manner. To a 2 ml of protein solution (containing about 10^{-7}–10^{-8} mol of sulfhydryl groups) buffered to pH 8 with 0.1 M phosphate or tris, 1.6 g of solid guanidinium chloride and 0.1 ml of 10^{-2} M solution of DTNB (adjusted to pH 6 with NaOH) were added and kept the solution at 25° for 5 minutes. Concentration sulfhydryl groups was calculated from the absorbance at 415 nm where the absorption due to thio-nitrobenzoate was maximum with ϵ=13,400.

Renaturation: Renaturation experiments were carried out in an open beaker dipped in a thermostated bath, or in a temperature controled closed vessel when an oxygen free condition was required. In the latter case, a nitrogen stream equilibrated with the same buffer solution as employed in the renaturation reaction at the same temperature was let into the reaction vessel and oxygen was purged enough time before the protein was added. Renaturation was started by adding a required amount of a stock solution of reduced protein (~3 mg per ml of 0.1 N acetic acid) to a tris buffer, 0.1 M, pH 8.2 with stirring magnetically. An aliquot was removed at an appropriate time interval to measure a sulfhydryl content and enzyme activity toward RNA as a substrate for which the original condition of Anfinsen *et al*[6] was followed. Concentration of the reduced protein was calculated on the basis of assuming the same ϵ as the original protein (ϵ=11,500). This assumption was based on the observation that the absorbance of the reoxidized protein was changed by insignificant amount from that of the starting solution of the reduced protein though the shape of the absorption spectrum was changed (λ_{max} for the reduced protein=275 nm).

RESULTS

Reduction of intramolecularly cross-linked RNase A with 2-thioethanol in the presence of 6 M urea gave the product which was shown to have 8 sulfhydryl groups by a DTNB titration. All four disulfide bonds originally present in the molecule were thus completely reduced. A couple among 8 sulfhydryl groups in the reduced protein seemed to be very readily oxidizable even under the most unfavorable condition for such a reaction; two sulfhydryl groups diminished after storage of the reduced protein solution in 0.1 M acetic acid in a refrigerator for 3 days.

Oxidation of the reduced protein by air in an open beaker was carried out at 25° in a 0.1 M tris buffer, pH 8.2, and a quite similar activity *vs.* time profile as established for the non-modified RNase A was obtained. A typical run was shown in Fig. 1. Concentration of the protein was chosen as low as possible (about 0.01–0.02 mg/ml) to eliminate a possibility that intermolecular disulfide bridges and therefore resulting polymeric forms might be produced. Measurement of thiol groups content reveals that about two cysteinyl

groups were lost in the initial few minutes, the result which might find an antecedent in the observation on the reduced protein as mentioned above. It should be noticed that the recovery of enzyme activity was remained only 70% even after 24 hours had ellapsed.

Figure 2 shows the result of the oxidation in an oxygen atmosphere under the same condition as for reactions in an open beaker. In one experiment, the reduced protein was added to a buffer saturated with oxygen. In another run, the reduced protein was added to a buffer under oxygen-free condition, incubated for 3.5 hrs, and then oxygen was introduced. Both experiments yielded the superimposable activity-time profile. Gradual expression of enzyme activity in an incubation period in a nitrogen atmosphere might be

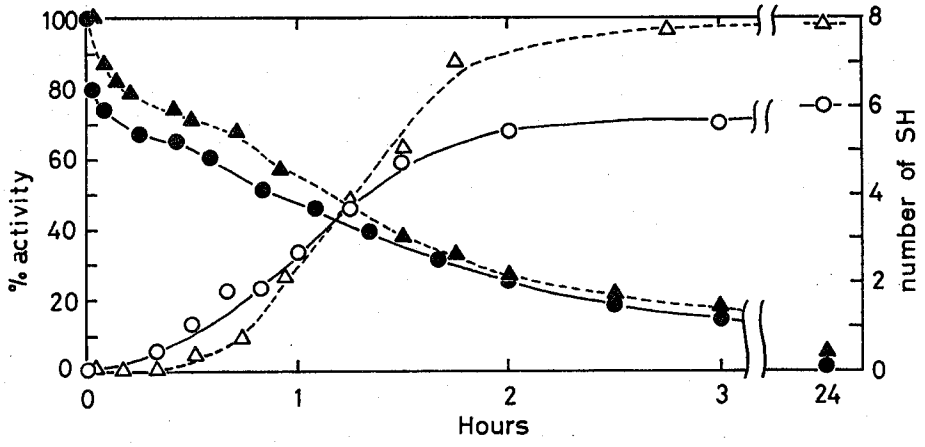

Fig. 1. Renaturation of reduced RNase A and reduced cross-linked RNase A in an open beaker. Open circles and triangles represent % enzyme activity toward RNA, scaled on the left side. Filled ones correspond to the thiol content whose scale is shown on the right side. Circles connected by a solid line are for the cross-linked RNase A, triangles connected by a broken line for RNase A.

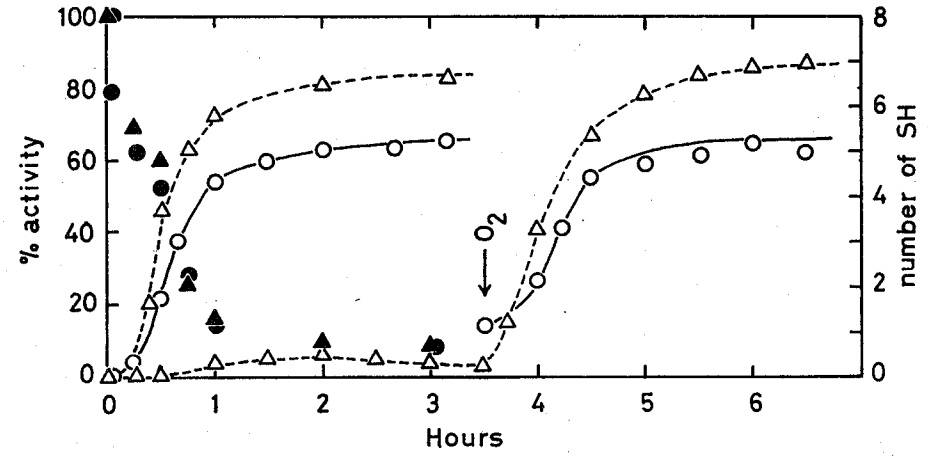

Fig. 2. Renaturation of reduced RNase A and reduced cross-linked RNase A in an oxygen atmosphere. Open (% enzyme activity) or filled (thiol content) circles and triangles are cross-linked RNase A (solid line) and RNase A (broken lines), respectively. Curves appeared in the left half of the figure are born of the renaturation experiments carried out in an oxygen saturated medium, those in the right half are of experiments in which the solutions were first kept in an oxygen free medium and oxygen was introduced at the point shown by an arrow.

attributed to a residual presence of oxygen which could not be purged off completely. Comparison of Figs. 1 and 2 showed that reoxidation in an oxygen atmosphere was evidently more accelerated than reactions in air, and furthermore, there were no appreciable differences in reoxidation pattern between the reduced RNase A and the reduced modified RNase A except final value of % activity regained.

DISCUSSIONS

In a folding process of protein into an active form, starting from a nascent polypeptide chain synthesized on ribosomes *in vivo*, or from an artificially denatured state of a native protein *in vitro*, formation of secondary structures might be considered far more rapid than that of higher structures because a secondary structure is mainly held by side chain interactions in close proximities and thus is determined by an amino acid sequence. On the other hand, higher structures might require much more time until they find a potential energy minimum which corresponds to the conformation of a native protein polypeptide chain since such higher structures are governed by interactions between distant portions of a polypeptide chain and there will be many ambiguous local minima of conformation energy as a result of long range interaction. A general hypotheses of protein folding is that rapidly folded secondary structures in a polypeptide chain would serve as nuclei to afford the final protein structure and remained without significant change in conformations.[7] In this experiment, our denatured protein, intramolecularly cross-linked RNase A was chosen from the reason that a cross-linking could play as to freeze a part of the original protein conformation after all disulfide bonds were disrupted and opened. Experiments showed the modified protein was actually renatured after reoxidation in some extent (75%) but not in full. Almost all cysteinyl residues disappeared after 4 hrs, leaving no possibilities that partial renaturation was due to an incomplete oxidation which was caused by the presence of a cross-linkage. The facts that the regained activity was not 100% and an absence of acceleration in a time course of renaturation compared with the non-modified RNase A suggest a local "frozen" conformation in the original protein played unimportant role in the refolding process (perhaps we should add "in this case") and some entanglings of the polypeptide chain were occurred in the process. An intramolecular cross-linkage does interfere a passway to a correct conformation by trapping some entangled molecules and delivering wrong ones. Secondary structures once formed in the initial step of renaturation will not be held rigid, since wandering of a molecular conformation in a wrong way could be rescued by a partial destruction of the initially formed local secondary structure.

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